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(54) Title: NOVEL RECOMBINANT VACCINIA VIRUS EXPRESSION VECTORS AND METHOD OF SELECTING SAME

(57) Abstract

Recombinant plasmids and vaccinia virus expression vectors which allow dominant selection have been made.

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NOVEL RECOMBINANT VACCINIA VIRUS EXPRESSION VECTORS AND METHOD OF SELECTING SAME

TECHNICAL FIELD

The present invention is related generally to construction of recombinant vaccinia virus vectors. More particularly, the present invention is related to the construction of unique vaccinia virus open-reading-frame expression vectors and a method for dominant selection of the same.

BACKGROUND OF THE INVENTION

Vaccinia virus is a useful vector for gene expression in mammalian cells. Advantages include the maintenance of infectivity, wide host range, large DNA capacity and correct synthesis, processing and transport of proteins. Because transcription of vaccinia virus genes is carried out by virus encoded enzymes in the cytoplasm and splicing of RNA does not occur, there are requirements for vaccinia promoters and uninterrupted open-reading-frames. In addition, the large size and lack of infectivity of the vaccinia virus genome prohibits the construction of recombinants by standard *in vitro* cloning techniques.

A two-step procedure has been developed to overcome these difficulties. In the first step, a plasmid is constructed that contains foreign gene(s) controlled by vaccinia promoter(s), flanked by sequences derived from a non-essential site on the vaccinia genome. In the second step, the foreign genetic material in the plasmid vector is inserted into the viral genome by homologous recombination in vivo.

However, selection or isolation of the recombinant virus thus produced, is not easily accomplished by the presently known techniques such as plaque hybridization, thymidine kinase (tk) negative selection, β-galactosidase expression and the like.

Of these, only insertional inactivation of the tk gene is a true selection step. Disadvantages of this method, however, include requirements for: inactivation of the viral tk gene which attenuates virus infectivity, use of special tk cell lines, and use of mutagenic selective agents, such as 5-bromodeoxyuridine. In addition, spontaneous tk mutants arise at a high frequency, necessitating additional steps to distinguish them from the recombinants.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a new recombinant vaccinia virus expression vector allowing dominant selection of the recombinant.

It is a further object of the present invention to provide a vaccinia virus recombinant expression vector the genome of which includes E. coli gpt gene allowing formation of the recombinant vaccinia plaques on a plurality of cell lines when replicated in a growth

medium comprising mycophenolic acid (MPA) and a substrate for purine metabolism.

It is another object of the present invention to provide a novel method for selecting recombinant vaccinia virus expression vectors containing a foreign gene which is desired to be expressed by said recombinant vaccinia virus.

Other objects and advantages of the present invention will become evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Fig. 1 demonstrates plaque formation of vaccinia virus in the presence and absence of MPA. Confluent BSCI cells were preincubated overnight (12-16 hours) in selective medium and subsequently infected with 1,000 PFU of vaccinia wild-type virus (A, B) or a recombinant virus that expresses the *E. coli* gpt gene (C, D). The cells were incubated for two days in the presence (A, C) or in the absence (B, D) of MPA, xanthine, and hypoxanthine and then stained with crystal violet.

Fig. 2 shows the results of genomic analysis of viruses from six randomly picked gpt plaques. Southern blots were prepared from HindIII digested DNA. On the right side, the fragment sizes (in kbp) of a phage lambda HindIII digest are indicated by arrows. (A) The Southern blot was hybridized with a [^{32}P]dCTP labeled gpt gene specific probe. Lanes 1-6, DNA of BSCI cells infected

with virus clones No. 1-6; lane 7, DNA of uninfected BSCI cells; lanes 8 and 9 have 10 and 100 ng of vaccinia wild-type DNA, respectively. (B) The same Southern blot hybridized with a vaccinia virus tk gene specific probe is shown. In lanes 8 and 9, the vaccinia virus 5.1 kbp HindIII-J fragment is visible; this fragment contains the tk gene.

Fig. 3 shows schematic construction of the vectors. DNA sequences including the vaccinia tk gene, E. coli gpt gene, and plasmid vector are indicated by filled, empty, and cross-hatched regions on the diagrams, respectively. Arrows show the direction of transcription from the 11 K (P11), the 7.5 K (P7.5), and tk gene promoters. The pUC sequences contain the ampicillin resistance gene and the origin of bacterial replication. The unique cloning sites downstream of the 11 K gene initiation codon are indicated.

Fig. 4 shows the sequence of the multiple cloning sites downstream of the 11 K gene initiation codon. The frameshift mutations were included by inserting additional G residues (arrows) downstream of the ATG codon. Note that only the restriction sites EcoRI, SalI, HincII, AccI, BamHI, and HpaI are unique. The initiation and the termination codons are boxed.

Fig. 5 shows the results of β-galactosidase expression in cells infected with the recombinant viruses. Confluent CVI cells (2.5×10^6) were infected with 7.5 PFU/cell of the indicated virus; after about 24 hour incubation, cytoplasmic extracts were prepared and the protein content and specific β-galactosidase activity were determined. The virus vF1sB was derived from the vector pTKgpt-F1s; the virus voF1sB is a derivative of pTKgpt-oF1s; and vtat was derived from pSC11. T7 refers to the viruses vTF7-3

(this virus expresses the bacteriophage T7 RNA polymerase) and vTFgal2 (a virus expressing the lacZ behind the T7 promoter); cells were infected with 7.5 PFU/cell of each of the two viruses. Prior to infection, the titers of all viruses were re-determined. β-galactosidase activities are mean value of two independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by a method for dominant selection including a vaccinia recombinant expression vector comprising in genome thereof an E. coli gpt gene and one or more foreign genes desired to be expressed by the recombinant virus, said recombinant virus forming plaques on a plurality of infectable cell lines when replicated in a growth medium comprising sufficient amount of mycophenolic acid to inhibit purine metabolism in the presence of sufficient amount of an unphosphorylated purine substrate.

The vectors of the present invention include in the genome a promoter that provides high level of expression and may include translation initiation and termination codons, and multiple restriction sites in three different frames which permit expression of partial or complete foreign genes.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials

are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques employed herein are standard methodologies well known to one of ordinary skill in the art.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases and low melting agarose were obtained from Bethesda Research Laboratories. T4 polymerase was from Pharmacia. Enzymes were used according to the instructions of the suppliers. MPA was from CalBiochem. Xanthine and hypoxanthine were from Sigma Chemicals. MPA and xanthine were dissolved in 0.1 N NaOH, hypoxanthine was dissolved in water and sterile filtered; the solutions were stored frozen as 10 mg/ml stocks.

Virus and cells. Vaccinia virus (strain WR) was originally from the American Type Culture Collection, replicated in Hela cells, and purified by standard techniques (Macket, et al., DNA Cloning: A Practical Approach, "The Construction and Characterization of Vaccinia Virus Recombinants Expressing Foreign Genes", pp. 191-211, IRL Press, Oxford. 1985). Human tk 143 cells were grown in Eagle's medium with 10% fetal bovine serum (FBS). CVI and BSCI cells were grown in Dulbecco's modified medium (DMEM) containing 10% FBS.

Formation of gpt recombinant virus. Recombinant viruses were prepared by standard procedures as described by Macket, et al., supra with the following modifications: 5 X 10^6 CVI cells (confluent monolayers) were infected with 0.2 plaque forming units

(PFU) of vaccinia virus per cell. Two hours after infection, 1 ml of a calcium DNA precipitate (consisting of 5 μg of supercoiled plasmid DNA, 1 μg of vacinia virus DNA, and 14 μg of sheared herring sperm DNA) was added to the cells. After 15 minutes of incubation at room temperature, 9 ml of medium (DMEM, 8% FBS with penicillin and streptomycin) were added. The medium was changed after 4 h and the incubation was continued for another 36 to 48 hours. Virus stocks were prepared by resuspending the infected cells in 1 ml of medium, freezing and thawing three times.

Selection of gpt virus. For the isolation of gpt recombinants, a plaque assay on BSCI cells was done as follows: confluent BSCI cells were preincubated in the gpt selection medium (DMEM, 2.5% FBS, 25 μg/ml MPA, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine) for 14 to 24 hours. The virus stock was digested with an equal volume of trypsin (0.25 mg/ml) for 30 minutes at 37°C and sonicated for 20 seconds on ice. Dilutions (10^{-3}, 10^{-4}, and 10^{-5}) of the trypsinized virus stock were used to infect the BSCI cells. After 1.5 hours of incubation at 37°C, the cells were overlaid with the gpt-selective medium containing 1% of low melting agarose. After 2 days of incubation, the cells were stained with neutral red (Gibco). The plaques were readily visible after overnight incubation.

Preparation and analysis of DNA. Recombinant plasmids were constructed and isolated by standard methodologies as described by Maniatis, et al., Molecular Cloning, Cold Spring Harbor Labs, Cold Spring Harbor, New York, 1982. For the genomic analysis of recombinant viruses, 2.5 x 10^6 BSCI cells were infected with the

material obtained from a single plaque and grown for 24 hours in selective medium. Total cellular DNA was extracted, digested with restriction endonuclease HindIII, electrophoresed through a 1% agarose gel, and subjected to Southern blot analysis following standard procedures.

Construction of recombinant plasmids. pTK61-gpt: HindIII linkers were added to the HindIII-HpaI fragment of E. coli gpt gene obtained from plasmid pSV2-gpt. Subsequently, the fragment was inserted into the unique HindIII site of pGS61, resulting in the plasmid pTK61-gpt.

pP11: The HindIII and SstI site, flanking the 11 K gene promoter in plasmid pSC42, were converted into XhoI sites by T4 polymerase treatment and ligation of XhoI linkers. The plasmid pSC42 contains the ClaI-EcoRI 11 K gene promoter fragment cloned into the SphI site of pUC19.

pTKgpt-F1s and pTKgpt-oF1s: The construction of these plasmids is outlined in detail in Figure 3.

pTKgpt-F2s and pTKgpt-F3s: The frameshift mutations were done by standard oligonucleotide directed mutagenesis. To obtain pTKgpt-F2s, a 30-mer oligonucleotide (5'-GACCTGCAGGAATTCCATTTATAGCATAGA-3'), and to obtain pTKgpt-F3s, another 30-mer (5'-ACCTGCAGGAATTCCCA-
TTTATAGCATAGA-3'), were used. Screening of the mutants was done by standard plasmid sequencing (Hattori, et al., Anal. Biochem. 152:232-240, 1986) with the help of the 20-mer primer (5'-GCGATGCTACGCTAGTCACA-3') derived from

the upstream region of the 11 K gene promoter (nucleotides -96 to -76 upstream of the initiation codon). The primary structure around the 11 K promoter region and the unique cloning cites in all vectors were confirmed by standard plasmid sequencing.

pTKgpt-F1sB and pTKgpt-oF1sB: The BamHI fragment of pMC1871 containing the E. coli lacZ gene (Shapira, et al. 1983, Gene 25:71-82) was cloned into the BamHI sites of pTKgpt-F1s and pTKgpt-oF1s.

A deposit of pTKgpt-F1s, pTKgpt-F2s and pTKgpt-F3s has been made at the ATCC on March 18, 1988 under accession numbers 67,655, 67,656 and 67,657, respectively. The deposits shall be viably maintained, replacing if it became non-viable, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and made available to the public without restriction in accordance with the provisions of the law. The Commissioner of Patents and Trademarks, upon request, shall have access to the deposit.

pTKgpt-F2sB: The SmaI-SalI fragment of pMC1871 was inserted into the HincII site of pTKgpt-F2s.

pTKgpt-F3sB: The SalI fragment of pMC1871 was cloned into the SalI site of pTKgpt-F3s.

Mycophenolic acid inhibition of the growth of vaccinia virus. The mycotoxin MPA inhibits the enzyme inosine monophosphate dehydrogenase and thereby prevents the formation of xanthine monophosphate. This results in the intracellular depletion of purine nucleotides and in

an inhibition of cell growth. Treatment of host cells with MPA, therefore, severely inhibits the growth of viruses. This was established by testing the effect of increasing amounts of MPA on the plaque formation of vaccinia virus. It was found that 25 μg/ml of MPA in the medium results in a nearly complete inhibition of plaque formation in all cell lines tested (BSCI, CVI, and human tk^- 143 cells). In BSCI and CVI cells, only a few tiny plaques could be observed on crystal violet-stained monolayers after 2 days of incubation (Fig. 1A). Replacement of the selective medium with normal medium resulted in plaque formation comparable to the control (Fig. 1B), indicating that the inhibition is reversible.

Expression of the E. coli gpt gene and its effect on plaque formation in the presence of MPA. The inhibition of the de novo synthesis of purines by MPA can be overcome by a cell that expresses the E. coli gpt gene, which codes for the enzyme xanthine-guanine-phosphoribosyl-transferase (XGPRT), in the presence of a substrate for purine metabolism such as xanthine and hypoxanthine in the growth medium. To determine whether the block of purine synthesis by MPA can also be overcome by a recombinant virus expressing the bacterial XGPRT, first the plasmid pTK61-gpt was constructed. In this construct, the gpt gene is controlled by the promoter from the vaccinia virus 7.5 K gene and is flanked by viral tk sequences. The 7.5 K gene promoter was chosen because it is active early and late in infection and might provide continuous production of the bacterial purine salvage enzyme. The plasmid was transfected into CVI cells that were infected with wild-type virus so that the gpt gene would be recombined into the viral tk locus. Putative recombinants were detected by a plaque

assay on BSCI cells in the presence of MPA, xanthine, and hypoxanthine as described herein. Large plaques formed only when the gpt gene was used for transfection, indicating that the desired recombinants behaved in the desired manner. One of the recombinants was plaque purified twice under selective conditions and a small virus stock was grown. A plaque assay demonstrated that this recombinant, in contrast to the wild type virus, formed plaques on BSCI cells in the presence of selective medium (Fig. 1C).

The genomic analysis of virus grown from six randomly picked plaques that formed during the first selection step is shown in Fig. 2. The HindIII fragments of all six genomes contain the anticipated 2.0 kbp fragment that hybridizes with the gpt probe (Fig. 2A). After washing off the labeled DNA, the same Southern filter was hybridized to a vaccinia virus tk gene specific probe (Fig. 2B). The tk sequences can be detected as a large fragment (4.7 kbp) and a small one (1.0 kbp), indicating the integration of the gpt gene into the viral tk locus. Since all plaques picked after the first selection step have integrated the selective marker, no other screening procedures are necessary to identify a viral recombinant. Thus the present invention provides a single step procedure for selection of dominant recombinants.

Construction of the insertion and expression vectors pTKgpt-F1s, pTKgpt-F2s, and pTKgpt-F3s. A series of plasmids was constructed that use the gpt gene as a selective marker and that allow the expression of foreign genes controlled by the promoter of the major late 11 K polypeptide (Fig. 3). The 5' regulatory region of the 11 K gene lies within a 30 kbp segment located immediately

upstream of the ATG initiation codon. Since the initiation codon forms part of a highly conserved TAAATG sequence within which the 5' ends of late mRNAs map, this region was chosen not be altered. The presence of an EcoRI site immediately downstream of the ATG facilitated the insertion of a polylinker with multiple unique cloning sites. Three vectors in which 0, 1, or 2 guanosine residues follow the ATG allow any coding sequences to be inserted in the correct reading frame. These vectors also provide all-frame stop codons at the end of the polylinker. The sequences downstream of the 11 K gene initiation codon for the three vectors (termed pTKgpt-F1s, pTKgpt-F2s, and pTKgpt-F3s) are shown in Fig. 4. The vector pTKgpt-oF1s (Fig. 3) is the orientation isomer to pTKgpt-F1s and has, therefore, the same sequence downstream of the 11 K gene ATG as pTKgpt-F1s.

Formation of recombinant vaccinia viruses that express β-galactosidase. To ensure the normal functioning of the vector constructs and to be able to easily quantitate the amounts of protein expressed, E. coli lacZ gene fragments that lack their own initiation codons (but still have their own termination codons) were inserted in frame into an appropriate restriction site of each of the four vectors. Four plasmids were obtained that were termed pTKgpt-F1sB, pTKgpt-F2sB, pTKgpt-F3sB, and pTKgpt-oF1sB. These plasmids were employed to construct viral recombinants. In each case, all the plaques obtained under selective conditions were able to convert the XGal in an agar overlay into its blue hydrolysis product. This indicated the co-expression of the selected marker gene and the gene of interest and shows that the lacZ gene fragments were in the predicted reading frames.

To quantitate the amounts of β-galactosidase produced by the viral recombinants, two gpt+ viruses, derived from the vectors pTKgpt-F1s and pTKgpt-oF1s, were plaque purified three times and small stocks were grown in CV1 cells. These stocks were used to infect CV1 cells at a multiplicity of 7.5 PFU of the respective virus. For comparison, the same assay was done also with a virus (vtat) based on the vector pSC11 that also expressed the lacZ gene driven by the 11 K gene promoter (but the β-galactosidase of which has a slightly different N-terminus), and with a vaccinia virus-T7 RNA polymerase hybrid system that expresses the lacZ gene behind the phage T7 promoter after co-infection with a T7 polymerase producing virus. The results of this analysis are shown in Fig. 5. The viruses based on the vectors pTKgpt-F1s, pTKgpt-of1s, and pSC11 express similar amounts of β-galactosidase, indicating that lacZ gene activity is relatively independent of the orientation and of the kind of neighboring sequences in the virus. The specific activity of pure β-galactosidase is 300,000 units/mg. Based on this number, the bacterial enzyme produced by virus-infected cells is more than 3% of the total cellular protein, an amount that is easily detectable in a Coomassie blue stained gel. In fact, a strong band in the 100,000 kDa range was observed upon electrophoresis of proteins from cells infected with a pTkgpt-F1s based virus, and not in the proteins of wild-type virus infected cells. The level of β-galactosidase expression using the 11 K promoter was about 2-fold higher than that achieved with the hybrid vaccinia virus-T7 RNA polymerase system under the infection conditions described in Fig. 5.

In summary, in accordance with the present invention, the gpt gene is incorporated into a plasmid

vector that has a vaccinia promoter and unique restriction endonuclease sites for insertion of a foreign gene. Because of vaccinia derived flanking sequences, the entire selection-expression cassette is inserted as a unit into the vaccinia virus genome by homologous recombination. Thus, all of the gpt^+ recombinants analyzed also contain the foreign gene that has been inserted into the plasmid vector. For convenience, the flanking sequence used in this study were derived from the vaccinia tk gene; however, since tk selection is no longer required in accordance with the method of the present invention, any non-essential site in the vaccinia genome can be employed.

In order to achieve high levels of expression, the promoter chosen for the vectors was derived from the major 11 k structural protein. However, other promoters well known to one of ordinary skill in the art could also be used. Although the mechanism of late transcription is still poorly understood and involves the attachment of a unique 5' poly(A) leader, the important sequences are contained within a relatively small region starting about 30 bp upstream of the RNA start site and include the translation initiation codon which is a part of the conserved TAAATG sequence. For this reason, the insertion sites for foreign genes were placed just downstream of the ATG. Since the vectors may prove useful for expressing open-reading-frames, multiple cloning sites were engineered in all three frames as well as termination codons. The efficacy of the system was illustrated by expression of β-galactosidase; the yield of enzyme was found to be greater than about 3% of the total cell protein. This expression level is higher than that obtained using the more widely used 7.5 K promoter and exceeded even that obtained with the recent

bacteriophage T7 vaccinia hybrid system (Fuerst, et al., Mol. Cell. Biol., 7:2538-2544, 1987). The vectors described herein could, of course, also be employed for direct cloning and expression of open-reading-frames in mammalian cells as well, in a manner similar to that used routinely with bacteriophage λ in E. coli.

Clearly, the gpt selection provides a number of important advantages over previous procedures devised to isolate recombinant vaccinia viruses. These include one-step plaque isolation without need for enrichment, application to a variety of cell lines, use of alternative insertion sites in the vaccinia genome, and absence of spontaneous selectable mutants. In addition, 5-bromodeoxyuridine, which is used for tk selection, is highly mutagenic, whereas mycophenolic acid is non-mutagenic in the Ames test and in the related SOS test. Avoidance of mutagens ensures virus stability. It is noted that gpt selection method could also be used with other virus vectors, including other members of the poxvirus family, herpesviruses, adenoviruses, retroviruses, and baculoviruses.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

WHAT IS CLAIMED IS

1. An expression vector, comprising a recombinant vaccinia virus in genome of which is incorporated an E. coli gpt gene and one or more foreign genes desired to be expressed by the recombinant virus, said recombinant virus forming plaques on a plurality of cell lines when replicated in a growth medium comprising sufficient amount of mycophenolic acid to inhibit purine metabolism in the presence of sufficient amount of an unphosphorylated purine substrate.

2. The expression vector of claim 1 further comprising a strong late promoter flanked by nonessential vaccinia sequences and multiple restriction sites in different open frames for expression of partial or complete foreign genes.

3. A method for selecting recombinant vaccinia vectors, comprising allowing recombinant vaccinia vectors to replicate on an infectable cell line in a growth medium comprising sufficient amount of mycophenolic acid to inhibit purine metabolism in the presence of sufficient amount of an unphosphorylated purine substrate and then isolating plaque forming recombinants therefrom by convention techniques.

4. The method of claim 3 wherein said purine substrate is selected from the group consisting of xanthine, hypoxanthine and combination thereof.

5. A plasmid comprising vaccinia virus DNA containing an E. coli gpt gene controlled by vaccinia virus promoter and a second vaccinia virus promoter next to a restriction endonuclease site for insertion of a foreign gene.

6. The plasmid of claim 5 having the identifying characteristics of ATCC 67,655.

7. The plasmid of claim 5 having the identifying characteristics of ATCC 67,656.

8. The plasmid of claim 5 having the identifying characteristics of ATCC 67,657.

FIG. 1A

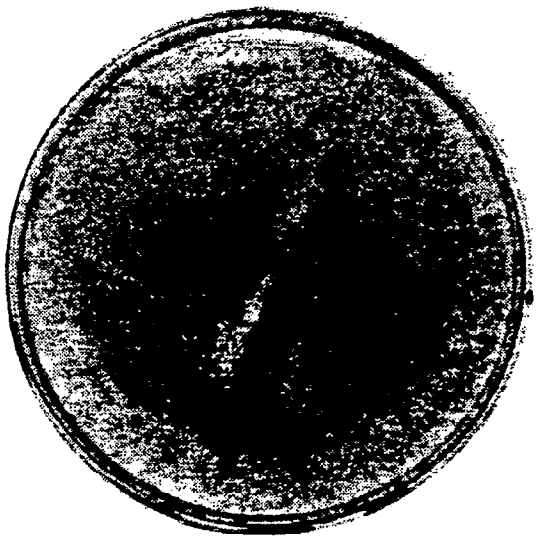


FIG. 1B

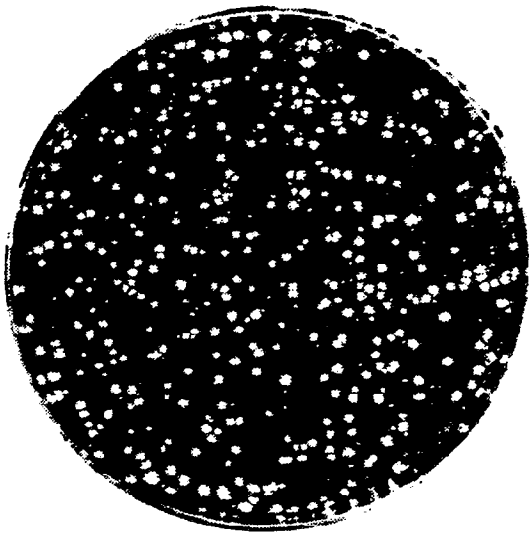


FIG. 1C

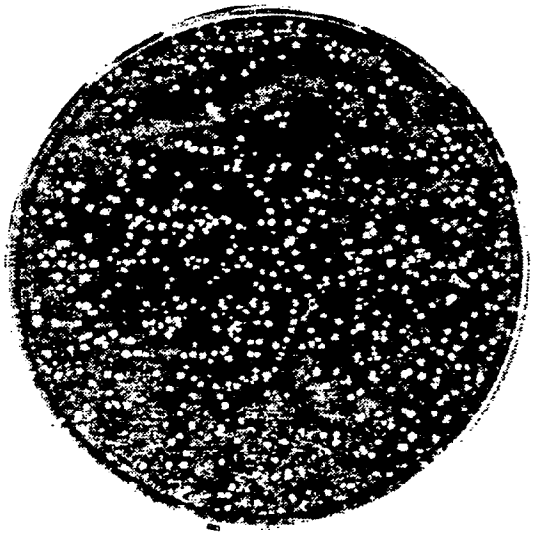


FIG. 1D

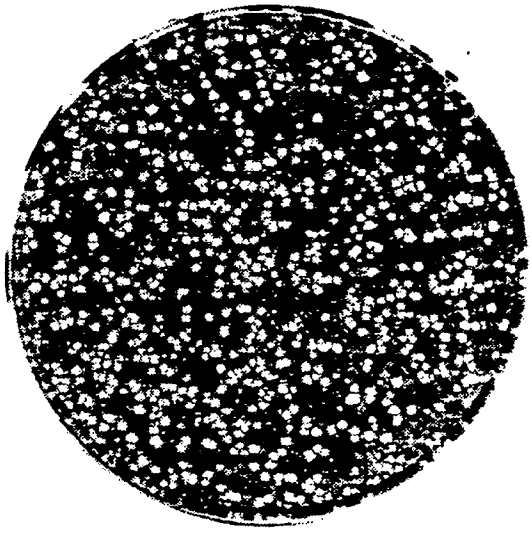


FIG. 2B

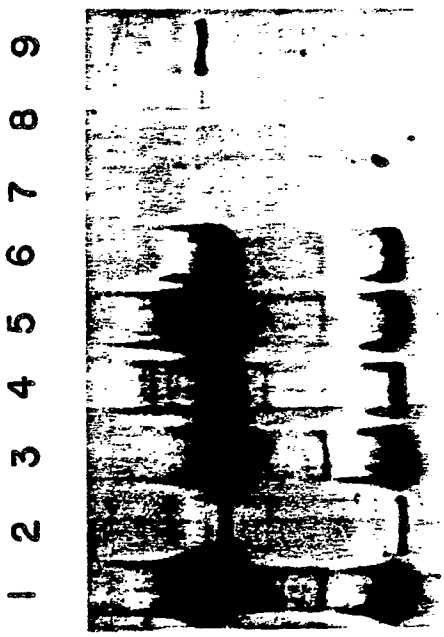
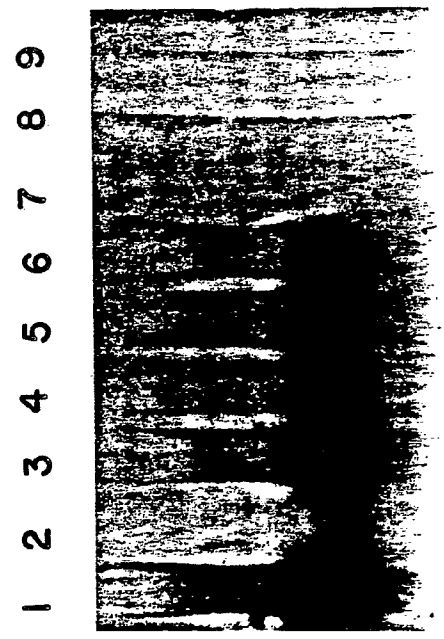


FIG. 2A



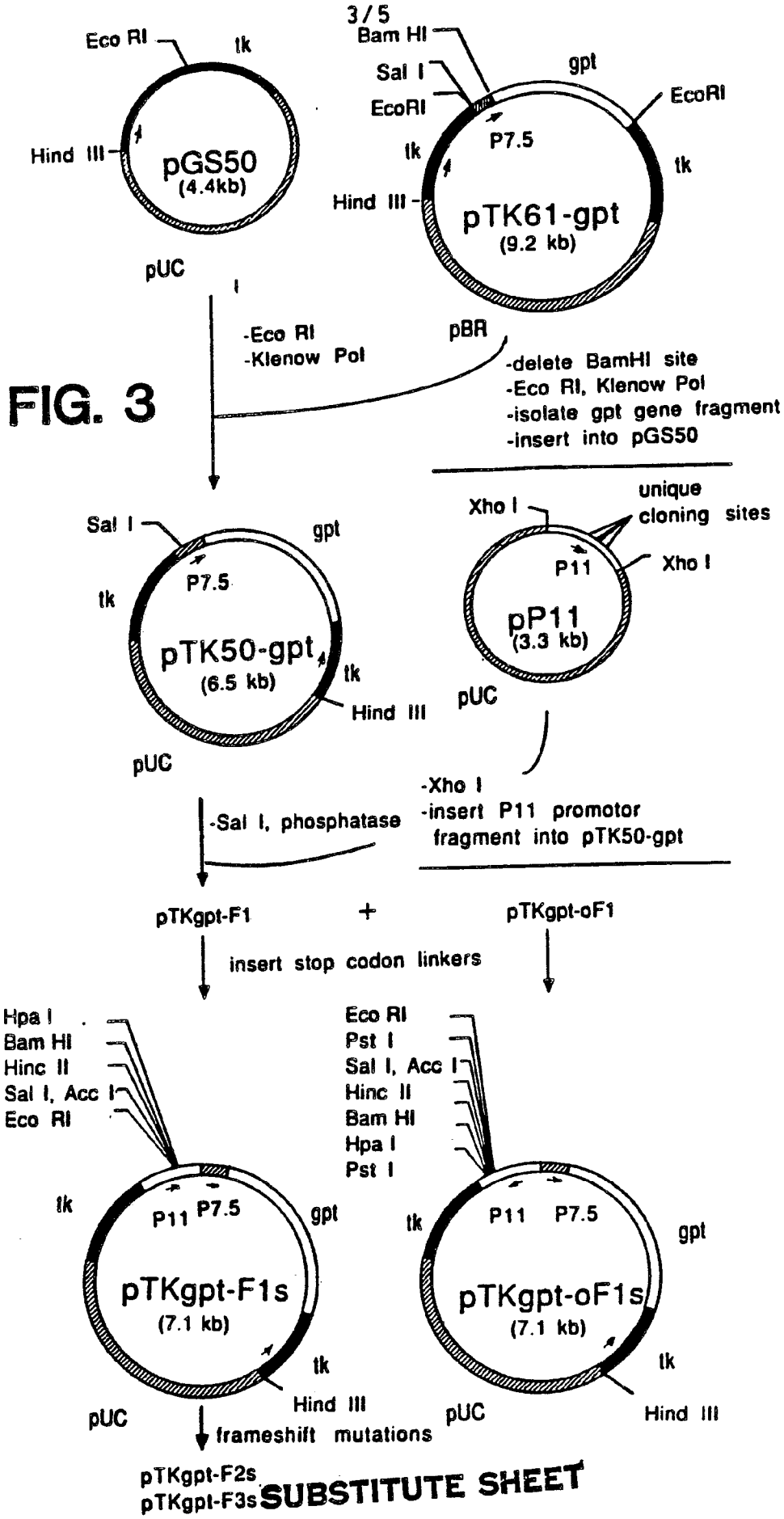
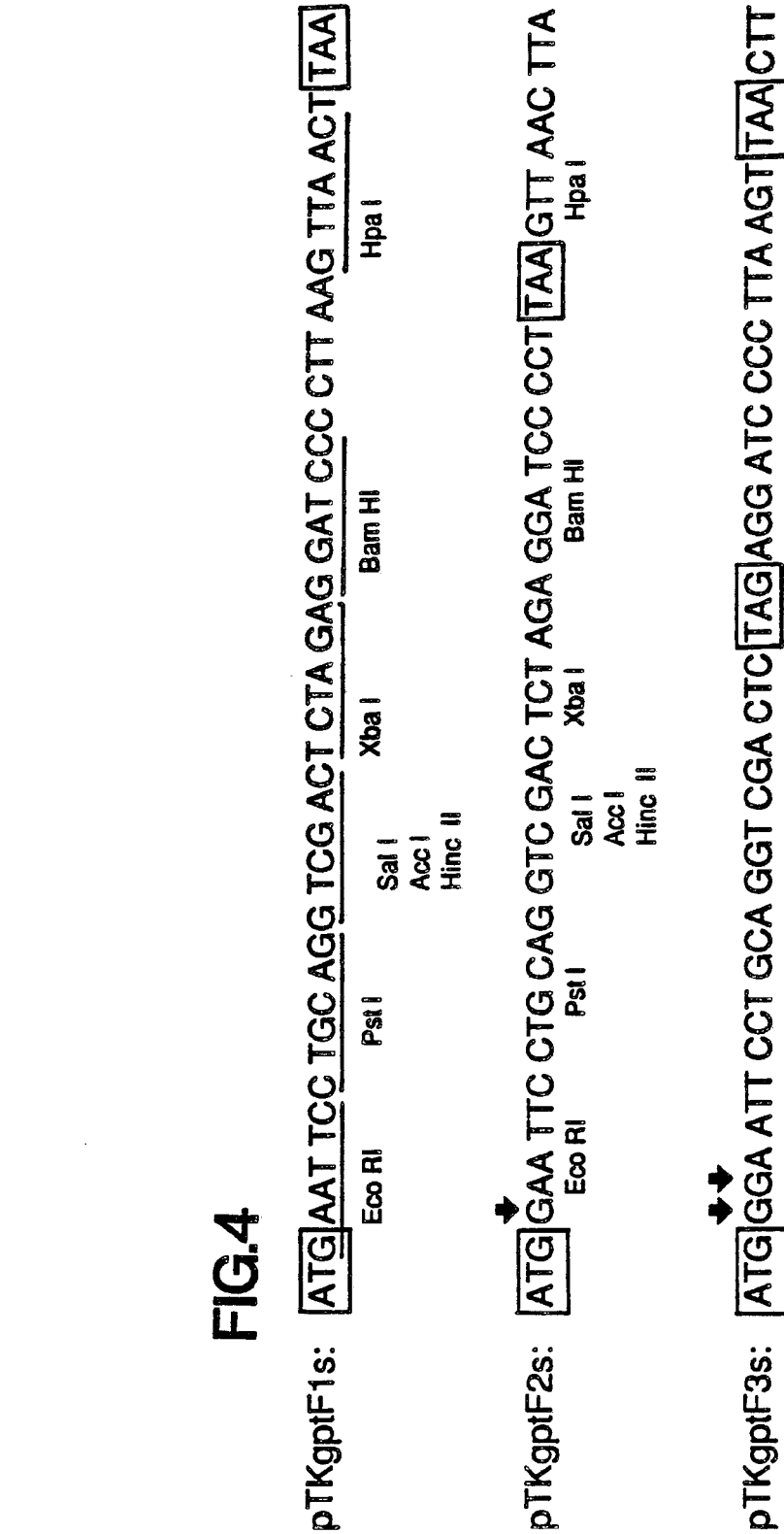


FIG. 3

FIG.4


pTKgptF1s:
ATG AAT TCC TGC AGG TCG ACT CTA GAG GAT CCC CTT AAG TTA ACT TAA
Eco RI
Pst I
Sal I
Acc I
Hinc II
Xba I
Bam HI
Hpa I
pTKgptF2s:
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Eco RI
Pst I
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Acc I
Hinc II
Xba I
Bam HI
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Bam HI
Hpa I

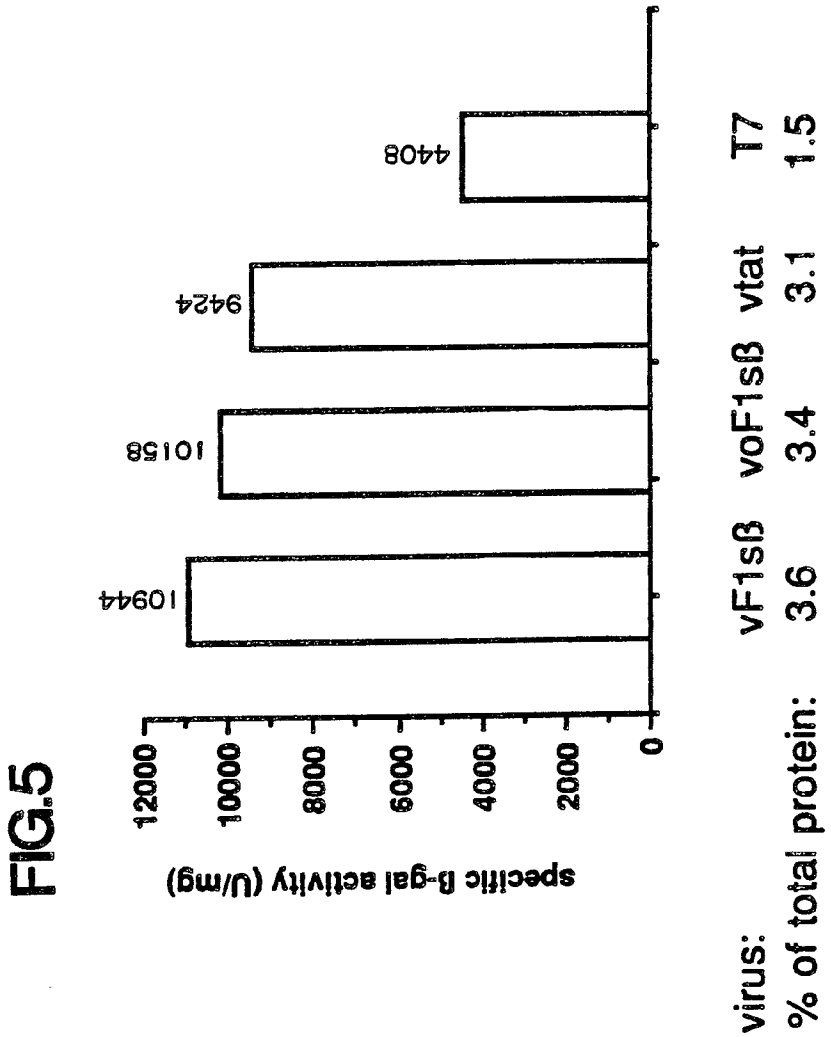
specific β-gal activity (U/mg)
12000
10000
8000
6000
4000
2000
0
10944
10158
9424
4408
virus:
% of total protein:
vF1sß 3.6
voF1sß 3.4
vtat 3.1
T7 1.5

FIG.5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00931

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/48, 1/70; C12N 7/00, 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5, 15, 172.3, 320

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CAS FILE 1967-1989
BIOSIS FILE 1967-1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y,P	Gene, Volume 65, No. 1, published 15 May 1988. D.B. Boyle, et al. "A dominant selectable marker for the construction of recombinant poxviruses," pp. 123-128. See entire document.	1-8
X,P	Journal of Virology, Volume 62, No. 6, published June 1988. F.G. Falkner, et al. "Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading fram expression vectors," pp. 1849-1854. See entire document.	1-8
Y	Plasmid, Volume 18, No. 1, published July 1987. B. Shepard, et al. "Transient expression system to measure the efficiency of vaccinia promoter regions", pp. 16-23, see Abstract and Results Section.	1-8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 JUNE 1989	23 JUN 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	BETH A. BURROUS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Science, Volume 232, published 27 June 1986/ G. Thomas, et al. "Expression and cell type-specific processing of human proenkephalin with a vaccinia recombinant," pp. 1641-1643. See entire document.	1,2 5-8
Y	Proc. Natl. Acad. Sci. USA, Volume 78, No. 4, published April 1981. R.C. Mulligan, et al. "Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase," pp. 2702-2706. See entire document.	1,3-8
Y	Proc. Natl. Acad. Sci. USA, Volume 82, No. 7, published April 1985. C. Bertholet, et al. "One hundred base pairs of 5' flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription", pp. 2096-2100. See abstract, Figure 2 and Results Section.	1-8
Y	Molecular and Cellular Biology, Volume 5, No. 8, published August 1985. C.A. Franke, et al. "Neomycine resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants", pp. 1918-1924. See entire document.	1-8